UbiQ
targeting the ubiquitin system

Activity-based Ub cascade profiling in intact cells

version V1, July-2016
applicable for UbiQ Triple E Probes, Activity-based probes for E1-E2-E3 enzymes
reference Mulder, M. P. C. et al. a cascading activity-based probe sequentially targets E1–E2–E3 ubiquitin enzymes. *Nature chemical biology.* **12,** 523–530 (2016). doi:10.1038/nchembio.2084.

protocol

electroporation of UbDha into living cells

1. HeLa cells cultured under standard growth conditions
2. transfected with GFP, GFP–UBE1, GFP–UBE2J1, GFP-tagged UBE2J1 or mutants C91A or C91S, and Flag-tagged UBE2J1 or mutant C91A using Effectene (Qiagen), according to the manufacturer's instructions.
3. the growth medium was refreshed 4–6 h after transfection and again 1–2 h before electroporation. To facilitate the incorporation of the probe.

For inhibition of UBE1,

1. cells were treated with 50 µM PYR-41 for 30 min prior to introducing the probe.
2. Following removal of the growth medium, cells were kept on ice for the duration of the protocol.
3. Cells were washed twice with cold electroporation buffer (2 mM HEPES, pH 7.4, 15 mM K2HPO4/KHPO4, 250 mM mannitol, 1 mM MgCl2).
4. 1.5 mL of a solution of the Cy5–UbDha, Cy5–Ub or rhodamine–UbDha probe in electroporation buffer (0.4 mg/mL) was added to each of the wells
5. electroporation was performed on ice using a Biorad GenePulser Xcell with CE and PE module Pulse Generator equipped with a Petri Pulser electroporation applicator (BTX)
    - settings: square wave, voltage=75 V, pulse length=3 ms, pulse interval=1.5 s, number of pulses=5, cuvette width=2 mm.
6. The electroporation applicator was turned 90 degrees, and electroporation was repeated once.
7. The probe solution was replaced by cold electroporation buffer, and cells were allowed to recover on ice for 2 min. After treatment, cells were washed twice with ice-cold PBS and allowed to recover under standard growth conditions as indicated (15–120 min).

For gel-based analysis,

1. samples were lysed using reducing SDS–PAGE loading buffer followed by
2. brief sonication and heating at 98 °C for 10 min before being
3. analyzed on SDS–PAGE gel,
4. visualized by fluorescence scanning scanning ($ë_{ex}$ = 625 nm; $ë_{em}$ = 680 nm (Cy5–UbDha) or $ë_{ex}$ = 473 nm; $ë_{em}$ = 530 nm (Rho–UbDha)).
5. Western blotting was performed as described above, and membranes were probed with rabbit anti-GFP serum[52] or mouse anti-Flag (1:1,000 dilution; Sigma-Aldrich, Sigma F3165).

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confocal microscopy

1. samples were fixed in 4% formaldehyde (Merck) in PBS
2. mounted onto glass slides (Thermo Scientific) using Prolong Gold mounting medium with DAPI (Invitrogen).
3. *Z* stacks of 5 images per cell were collected on a Leica SP5 confocal microscope equipped with HyD detectors, using a 63× magnification lens in combination with 2.5–4× digital zoom and represented as maximum *z* projections.
    a. Image processing and fluorescence intensity analysis were performed using ImageJ64 software,
    b. colocalization was expressed in the form of Mander's overlap coefficients calculated using JACoP.
    c. Pixel plot analyses were generated using Leica LASAF software.

statistics and reproducibility

Statistical data are presented as mean ± standard deviation (s.d.). Comparisons between the samples were made using a two-sided t-test (assessed against controls). Data analyzed were derived from at least two biologically independent experiments (n) as indicated in figure legends.